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I. Introduction and Background

A. Overview

This manual provides details and information necessary to use the miRANDA qPCR-Ready miRNA Array Kit to detect and quantify small non-coding RNAs from 18 separate Human tissue cDNAs. To ensure optimal results, please read the entire manual before using the reagents and material supplied with this kit.

B. Importance of MicroRNAs and Other Small Non-Coding RNAs

The field of non-coding RNAs has gained increasing attention in recent years, particularly due to the discovery of micro RNAs (miRNA). Micro RNAs are short (typically 19-24 nucleotides) single stranded RNAs that regulate the expression of target genes by interacting with complementary sites in the 3' UTR of the target mRNAs and inhibiting translation. miRNAs are a conserved group of non-coding RNAs with very important regulatory roles.

Mature miRNAs are excised from stem-loop precursors, which are themselves transcribed as part of longer primary transcripts. These primary miRNAs appear to be first processed by the RNase Drosha in the nucleus, after which the precursor miRNAs are exported to the cytoplasm where the RNase Dicer further processes them. These enzymes are also involved in the generation of mature small inhibitory RNAs (siRNA) from exogenously transferred double stranded siRNA precursors.

The current, standard method for detecting and quantifying novel miRNA molecules involves Northern blottting with hybridization. Detecting and quantitating known miRNAs can be done using pre-designed reverse priming and reverse transcription followed by primer sets built for the specific miRNA for Real-time PCR analysis. These sets require many steps and can take several hours to complete and trouble-shoot. The miRANDA kit provides immobilized, anchored-tailed small non-coding RNAs from 18 separte Human tissues in an array format. The user simply only needs to design the forward primer which corresponds to the miRNA of interest and provide Real-time PCR SYBR green master mix (for Real-time PCR) or typical PCR mastermix for End-point PCR expression profiling.

Recently, previously unknown germline specific classes of miRNA-like molecules were identified in mouse testes and mouse oocytes, illustrating the need for continued and in depth miRNA discovery efforts across a wide range of tissues. These facts taken together demonstrate an ever-increasing need for simple, robust, and sensitive methods that enable discovery and quantitation of microRNAs and their precursors.

C. Overview of Protocol

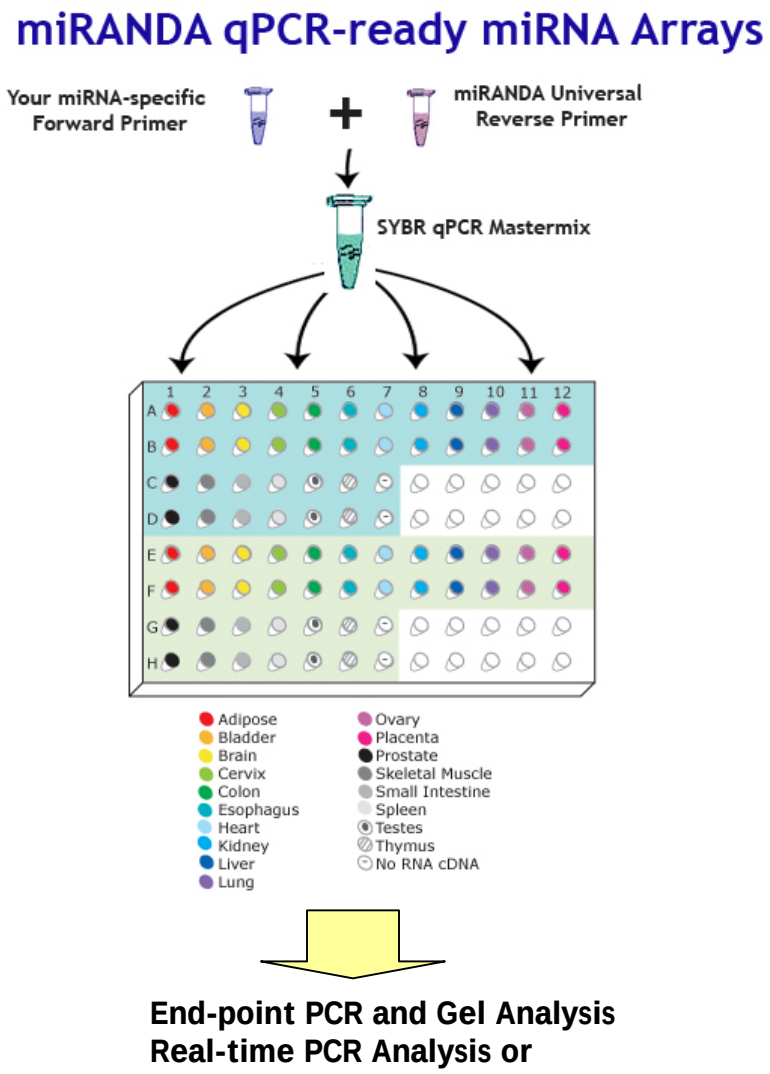


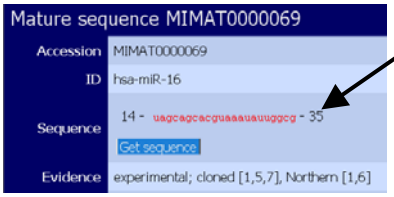
Fig. 1. Workflow schematic for a typical miRANDA MicroRNA qPCR Array experiment.

D. Primer Design Considerations

The user needs only to design the forward "sense" orientation primer to perform the end-point or qPCR reactions. MicroRNAs typically range in size from 19 – 24 nt. We recommend using the exact sequence of the miRNA being studied when designing the forward primer. If the miRNA under study is known and documented, using the miRBase database can be an easy starting point :
(http://microrna.sanger.ac.uk/sequences/search.shtml).

An example of the known and documented miRNA, Human miR-16, is shown below.

Hsa-miR-16



Simple: Directly use sequence of mature miRNA as forward primer in oligo design.

The mature miRNA sequence 5' – uagcagcacguaaauauuggcg – 3' can be simply converted to a DNA sequence and used directly as the forward primer for end-point and qPCR analysis.

Forward primer for hsa-miR-16:
5' – TAGCAGCACGTAAATATTGGCG – 3'
Tm= 58.9 °C, 45% GC and length =22 bases.

If the user is developing a new assay for a novel miRNA, follow the guidelines of the example above. Design the primer to have a Tm of at least 55°C and to have a length of at least 18 bases. If the primer being designed for the miRNA to be studied has a Tm below 55°C, lower the annealing temperature in your cycling conditions for end-point and qPCR instrument settings. An example of successfully using this approach is demonstrated in **Fig. 5**. Once the user has designed a suitable forward primer, we suggest performing an initial PCR test using the kit's Universal cDNA template and analyze PCR products using gel electrophoresis (see below in **Section E. Use of miRANDA Universal cDNA template**).

E. Use of the miRANDA Universal cDNA template

The miRANDA kit comes with a tube containing a pool of all 18 Human cDNAs lyophilized to allow for a primer design pre-test proceeding a full array experiment. Simply hydrate the miRANDA Universal cDNA template with 20 µl nuclease-free water and use 1 µl per 25-50 µl PCR reaction. The following PCR reaction conditions are recommended:

	1 µl miRANDA Universal cDNA template
	0.5 µl miRANDA Universal Reverse Primer (10 µM)
+	1 µl miRNA-specific Forward Primer (10 µM, user designed)
	2.5 µl 10X PCR Buffer with 2.5 mM $MgCl_2$
	20 µl nuclease-free water
	Total of 25 µl

Prepare reaction in a suitable PCR tube or plate and thermocycle as follows:

Heat denature at 95°C 10 min.

Heat denature at 95°C 15 sec.
Anneal Primers at 60°C, 1 min.] 30 cycles

Hold at 15°C (optional)

Prepare and pour a 3.5% agarose (3.5 g agarose in 1X TAE, boil; or 1X TBE) gel with a suitable stain (Ethidium Bromide, etc.). Add 2.5 µl of 10X Loading dye, mix and load 10 µl into a well of the gel. Also run a suitable DNA size marker (50-2,000 bp) along with your samples. Take care to only electrophorese your gels for 10-15 minutes and then visualize. An example of end-point PCR primer tests and gel electrophoresis is shown in **Fig. 3.**

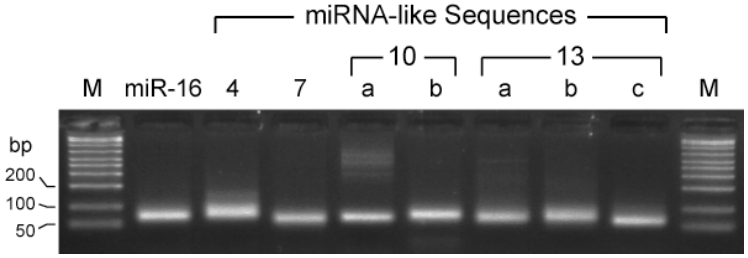


Fig. 2. miRANDA end-point PCR for miR-16 and seven newly identified miRNA-like sequences by SBI. Numbers on the top of the gel correspond to cDNA clone number. Letters indicate specific sequence found in corresponding clone. Include a DNA size ladder with markers in the range of 50-2,000 bp (*e.g.*, Bio-Rad AmpliSize™ DNA Ladder, Cat. # 170-8200). M denotes DNA Marker lanes.

F. List of Components

Each miRANDA qPCR-Ready MicroRNA Array Kit contains the following 4 components:

i. miRANDA cDNA array plate with lyophilized cDNAs in the well positions detailed below. The plate itself is an optical qPCR plate, suitable for use with Applied Biosystems' Real-time PCR systems 7300, 7500 and 7900 96-well format. An optical, adhesive cover is also provided.

ii. One 0.5 ml tube containing lyophilized miRANDA Universal cDNA template (resuspend with 20 μl nuclease-free water, enough for 20 PCR reactions.)

iii. One 0.5 ml tube containing lyophilized miRANDA Universal Reverse adapter Primer (resuspend with 50 μl nuclease-free water to make 10 μM concentration, enough for 100 reactions.)

iv. One 0.5 ml tube containing lyophilized Human U6 snRNA control Forward Primer (resuspend with 50 μl nuclease-free water to make 10 μM concentration, enough for 50 reactions.)

Each miRANDA qPCR-Ready MicroRNA Array Kit provides enough material to perform 20 Primer designs using the miRANDA Universal cDNA template with end-point PCR analysis. SBI's miRANDA MicroRNA qPCR Array includes 4 complete sets of 18 different Human cDNAs arrayed in the format detailed in **Fig. 1.** and listed in Table format below.

Format of Human cDNA arrangement on the miRANDA qPCR-Ready miRNA Array

	1	2	3	4	5	10	7	8	9	10	11	12
A	Adipose	Bladder	Brain	Cervix	Colon	Esophagus	Heart	Kidney	Liver	Lung	Ovary	Placenta
B	Adipose	Bladder	Brain	Cervix	Colon	Esophagus	Heart	Kidney	Liver	Lung	Ovary	Placenta
C	Prostate	Skeletal Muscle	Small Intestine	Spleen	Testes	Thymus	Water					
D	Prostate	Skeletal Muscle	Small Intestine	Spleen	Testes	Thymus	Water					
E	Adipose	Bladder	Brain	Cervix	Colon	Esophagus	Heart	Kidney	Liver	Lung	Ovary	Placenta
F	Adipose	Bladder	Brain	Cervix	Colon	Esophagus	Heart	Kidney	Liver	Lung	Ovary	Placenta
G	Prostate	Skeletal Muscle	Small Intestine	Spleen	Testes	Thymus	Water					
H	Prostate	Skeletal Muscle	Small Intestine	Spleen	Testes	Thymus	Water					

The array allows for the examination of 4 individual miRNA species across 18 tissues, 2 miRNA species across 18 tissues in duplicate or 1 miRNA species across 18 tissues along with the positive normalization transcript in separate wells for U6 snRNA across 18 tissues, both reactions performed in duplicate…your choice.

The kits are shipped dry and should be stored at ambient temperature. Once the miRANDA Universal cDNA template, Universal Reverse Primer and the Human U6 snRNA Control Forward Primer are resuspended, they should be stored at -20°C. Properly stored kits are stable for 1 year from the date received.

G. Additional Required Materials

- Nuclease-free water
- Thermocycler (with heated lid)
- Thermocycler PCR tubes or plates for end-point reactions
- PCR Mastermix, including *Taq* polymerase for PCR
- 3.0-3.5% Agarose Gel in Tris-Borate EDTA (TBE) or Tris-Acetate EDTA (TAE) Buffer
- DNA Size Ladder with markers from 50 to 2,000 bp (Bio-Rad AmpliSize™ DNA Ladder; Cat. # 170-8200)
- Real-time qPCR Instrument

IMPORTANT:

- Recommended 2X SYBR Green qPCR Mastermixes
  SBI has tested and recommends SYBR Green Master mix from three vendors: Power SYBR Master Mix® (Cat numbers 4368577, 4367650, 4367659, 4368706, 4368702, 4368708, 4367660) from Applied Biosystems; SYBR GreenER™ qPCR SuperMix for ABI PRISM® instrument from Invitrogen (Cat numbers 11760-100, 11760-500, and 11760-02K); and RT² Real-Time™ SYBR Green / ROX PCR (Cat numbers PA-012 and PA-112) from SuperArray.

II. Protocol: Real-time qPCR

For end-point PCR reactions, please refer to the Section IE above, "Use of the miRANDA Universal cDNA template".

A. qPCR Reaction Set up

To determine the expression profile for your miRNA under study, mix the following per well:

For Single well determination:

	12.5 μl	2X SYBR Green qPCR Mastermix buffer
+	1 μl	User-designed Forward Primer (10 μM)
	0.5 μl	miRANDA Universal Reverse Primer (10 μM)
	16 μl	Nuclease-free water
	30 μl	Total / well

We recommend making a SYBR Green qPCR Mastermix to evaluate the 18 tissue expression profile as follows:

For 18 tissue miRNA expression profiling with single well determination per tissue:

	250 μl	2X SYBR Green qPCR Mastermix buffer
+	20 μl	User-designed Forward Primer (10 μM)
	10 μl	miRANDA Universal Reverse Primer (10 μM)
	320 μl	Nuclease-free water
	600 μl	Total (enough for 20 wells with 30 μl/well)

The Mastermix contents can be scaled down or up depending upon on your experimental needs. Once reagents are loaded into array wells, cover the plate with the optical adhesive cover (provided) and spin briefly in a centrifuge to bring contents to bottom of wells. Place plate in the correct orientation (well A1, upper left) into the Real-time qPCR instrument and perform analysis run.

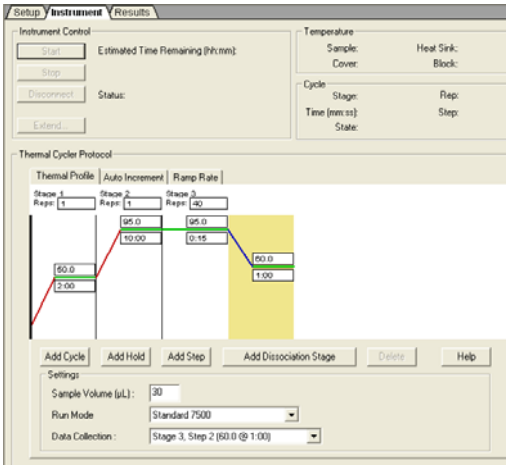
B. Real-time qPCR Instrument Parameters

Follow the guidelines as detailed for your specific Real-time instrumentation. The following parameters tested by SBI were performed on an Applied Biosystems 7300 Real-time PCR System but can also apply to an ABI 7500 or an ABI 7900 96-well system. The details of the thermocycling conditions used in testing at SBI are shown below. A screenshot from SBI's instrument set up is shown on the right. Default conditions are used throughout except for those cases where the Forward Primer's Tm is below 55°C, then the annealing temperature in Step 2 of Stage 3 is lowered from 60°C to 50°C.

qPCR cycling and data accumulation conditions:

1. 50°C 2 min.
2. 95°C 10 min.
3. 95°C 15 sec.
4. 60°C 1 min.

(40 cycles of steps 3 and 4), data read at 60°C 15 sec. Step (gold rectangle)



An additional recommendation is to include a melt analysis after the qPCR run to assess the Tm of the PCR amplicon to verify the specificity of the amplification reaction. Refer to the User Manual for your specific instrument to conduct the melt analysis and the data analyses of the amplification plots and Cycle Threshold (Ct) calculations. In general, Cycle thresholds should be set within the exponential phase of the amplification plots with software automatic baseline settings.

III. Quality Control and Sample Data

A. How the cDNAs are synthesized

All of the Human miRNA cDNAs are prepared by a 3' RACE method as illustrated in **Fig. 3.**

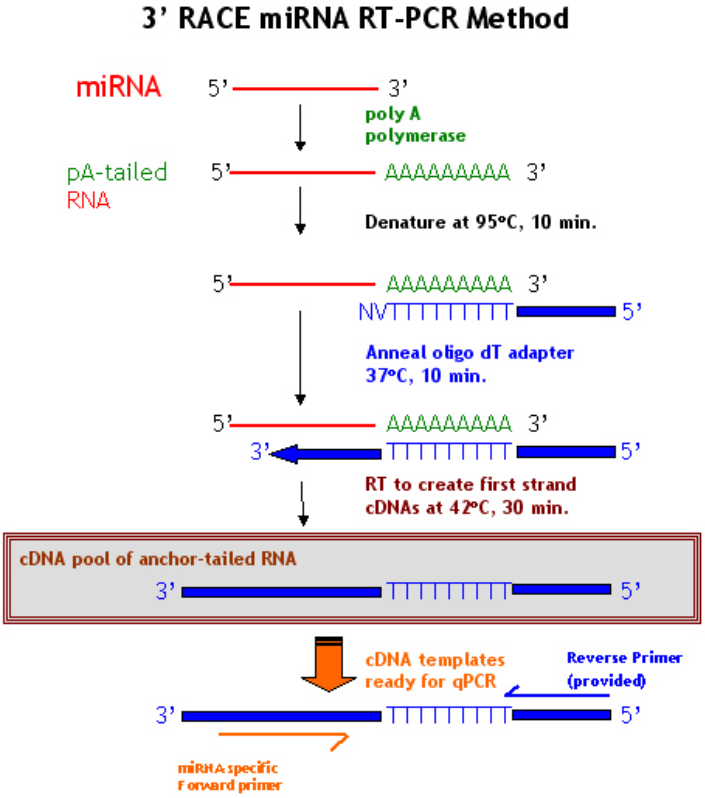
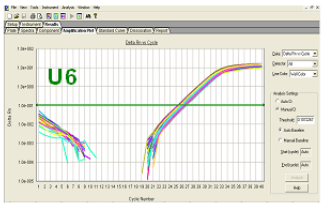
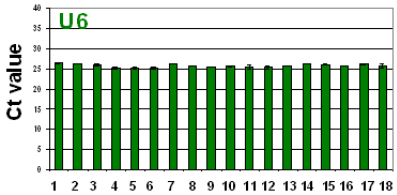


Fig. 3. Illustration of the 3' RACE method for the synthesis of miRNA cDNA sequences. The protocol follws the methods of Shi, R., Chiang, V.L., 2005.

B. miRANDA cDNA set Normalization

The anchor-tailed cDNAs are deposited then lyophilized on the array in the format depicted in **Fig. 1.** The sets are then normalized using a primer specific for U6 snRNA transcript (Control Forward primer supplied with kit) The array cDNA sets are quality-controlled using Real-time PCR and SYBR green reagents using the default parameters recommended by the manufacturer's specifications and described earlier in the manual.





Human U6 snRNA Control Forward Primer Sequence:
5' – CACCACGTTTATACGCCGGTG – 3'
Tm= 62°C, amplicon size=79 bp

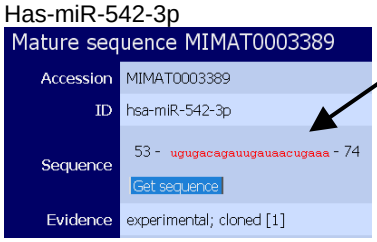
1	adipose	7	heart	13	prostate
2	bladder	8	kidney	14	skeletal muscle
3	brain	9	liver	15	small intestine
4	cervix	10	lung	16	spleen
5	colon	11	ovary	17	testes
6	esophagus	12	placenta	18	thymus

Fig. 4. Real-time qPCR normalization data using a forward primer specific for Human U6 snRNA (provided in kit) was used to detect and normalize the cDNA set. Quality control results for the balanced set are depicted. The Real-time amplification plots to the left and a bar graph of tissue cDNA and Ct values derived from the Real-time data with the U6 snRNA transcript is shown on the right. The sets are balanced to approximately ± 1 Ct of each other.

C. Specificity Tests

To assess the specificity and proper orientation of the miRNA array, oligonucleotide primers are synthesized both in the "sense" and the "antisense" orientation. An example for the known, documented miRNA miR-542-3p is detailed below.

Has-miR-542-3p



Sequence of mature miRNA as forward primer in "sense" oligo design, and then designed in the "antisense" oligo as control.

The mature miRNA sequence 5' – ugugacagauugauaacugaaa – 3' can be converted to a DNA sequence along with designing its complement, or "antisense" primer sequence.

Forward "sense" primer for hsa-miR-542-3p:
5' – TGTGACAGATTGATAACTGAAA – 3'

Forward "antisense" primer for hsa-miR-542-3p:
5' – TTTCAGTTATCAATCTGTCACA – 3'
Tm= 49.6 °C, 32% GC and length =22 bases.

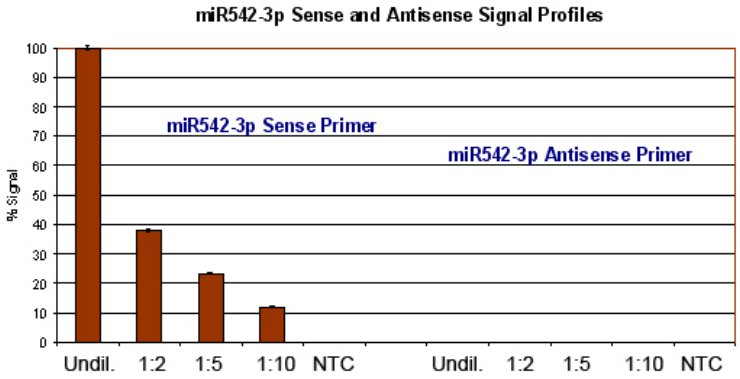
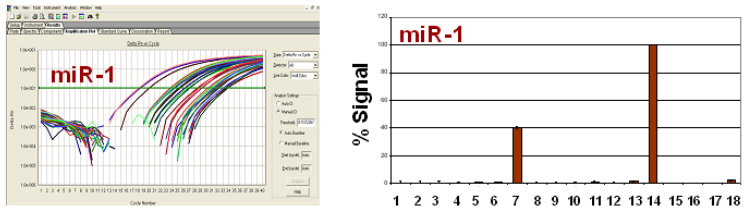


Fig 5. Sense and anitsense test of the miRANDA cDNA. Dilutions of the miRANDA Universal cDNA template as well as no template controls (NTC) were tested with either sense or antisense orientation for the miR-542-3p molecule. Quantitative results are observed for the "sense" orientation of miR-542-3p. No signals are observed in the "antisense" or no template controls. The annealing temperature for the qPCR cycling conditions was lowered to 50°C.

D. Sample Data

The cDNA sets were also tested with 2 primers specific for 2 known miRNA molecules: miR-1 (heart and skeletal muscle-specific) and miR-122a (abundant in liver). The amplification plots and corresponding expression bar graphs are shown in **Fig. 5**. **Panels A. and B.**

A.



B.

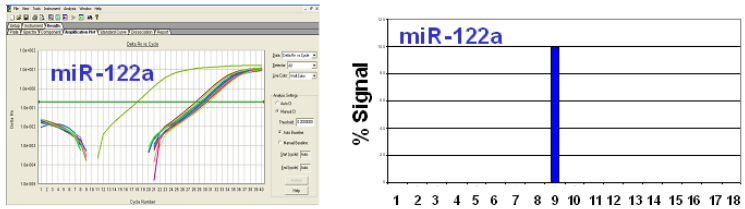


Fig. 5. Real-time qPCR data using primers specific for Human miR-1 (**Panel A**.) and for miR-122a (**Panel B**.). The amplification plots are shown on the left with the resulting expression profile bar graphs based on Ct values is shown on the right. The defualt qPCR cycling conditions were used with an annealing temperature of 60°C in Step 2 of Stage 3.

These two known miRNAs, miR-1 and mir-122a, have very specific tissue expression patterns. Real-time qPCR data confirmed that miR-1 is restricted to skeletal muscle and heart. The sensitivity of the assays also reveals very low but detectable signals in additional tissues. miR-122a is known to be highly abundant in liver.

IV. Troubleshooting

Problem	Possible Solution
No PCR bands using user-designed Forward Primer with miRANDA Universal cDNA template.	Alter thermocycle conditions, lower annealing temperature. Include the U6 control Forward primer in a separate reaction as PCR control.
Too many PCR bands using user-designed Forward Primer with miRANDA Universal cDNA template.	Alter thermocycle conditions, elevate annealing temperature. Include the U6 control Forward primer in a separate reaction as PCR control.
No qPCR signals using user-designed Forward Primer	Test U6 control Forward primer with Universal Reverse Primer on miRANDA Universal cDNA template on qPCR instrument.
Correct size band and larger PCR band(s) observed in end-point PCR tests.	Potential precursor pri-miRNA molecules are being amplified.

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VI. Related Products

- **miRANDA Universal miRNA cDNA template** (Cat. # RA650A-1)
  Pool of all 18 Human miRNA cDNAs (enough for 20 50 µl-reactions)
  A universal reverse adaptor primer (10 µM)
  A positive control forward primer (U6 snRNA, 10 µM)

- **MicroRNA Discovery™ Kit** (Cat. # RA410A-1)
  Rapid identification of new MicroRNAs and MicroRNA-like molecules. Amplification and cloning can be initiated in a single day (3 steps, 1 day.) The alternative method takes approximately 1 week (9 steps.)

- **Pre-Made MicroRNA-Enriched cDNAs** (Cat. # RA500A-1 – RA509A-1)
  Tissue-specific amplified cDNA generated by SBI using the MicroRNA Discovery™ Kit can be used for cloning microRNA.

- **Global MicroRNA Amplification Kit** (Cat. # RA400A-1)
  Simple amplification kit allows cDNA amplification for qRT-PCR and microarray studies from as little as 50 ng of starting total RNA.

- **Full Spectrum™ Complete Transcriptome RNA Amplification Kit** (Cat. # RA101A-1)
  The Full Spectrum RNA Amplification Kit provides an inexpensive method to amplify reverse transcribed RNA in a sequence independent, unbiased, and uniform manner with better representation of 5' end of mRNA sequences. This approach maintains the relative levels of each transcript in the starting mRNA samples—even when using starting amounts of RNA as low as 5ng or when using heavily degraded RNA.

- **Full Spectrum™ MultiStart Primers for T7 IVT** (Cat. # RA300A-2)
  Extract more data from your RNA than currently available primers in nearly all commercially-available T7 IVT kits using Full Spectrum™ technology. Just replace the existing T7 primer with the Full

Spectrum™ primers. Compatible with Affymetrix GeneChip® hybridization.

VII. Technical Support

For more information about SBI products and to download manuals in PDF format, please visit our web site:

http://www.systembio.com

For additional information or technical assistance, please call or email us at:

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Mountain View, CA 94043

Phone: (650) 968-2200
(888) 266-5066 (Toll Free)

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General Information: info@systembio.com
Technical Support: tech@systembio.com
Ordering Information: orders@systembio.com

VIII. Licensing and Warranty Statement

Limited Use License

Use of the miRANDA qPCR-Ready miRNA Array Kit (*i.e.*, the "Product") is subject to the following terms and conditions. If the terms and conditions are not acceptable, return all components of the Product to System Biosciences (SBI) within 7 calendar days. Purchase and use of any part of the Product constitutes acceptance of the above terms.

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